

Clorox Services company



TITLE: Sanitizing Properties of Automatic Toilet Bowl Cleaner

CLOROX STUDY NO: 5813-EX-093

CLOROX PROTOCOL NO: 6892-10

SPONSOR : Clorox Services Company
7200 Johnson Drive
Pleasanton, CA 94588-8004

Testing Sites: Lonza Research and Development
79 Route 22 East
Annandale, NJ 08801
(GLP Toilet Testing Laboratory)

Benchmark Laboratories
4777 Saucon Creek Road
Center Valley, PA 18034
(Microbiology Efficacy Testing Laboratory)

TYPE OF STUDY: This is a non-clinical laboratory study and will be performed according to FIFRA Good Laboratory Practice Standards (40 CFR 160). All methods not specifically described in this protocol will be performed according to referenced Standard Operation Procedures (SOP) for the laboratories involved in the study.

PURPOSE: The objective of this study is to develop data to establish a sanitizing claim for an in-tank automatic toilet bowl cleaner in toilet bowl water. Additionally, the longevity of the test substance will be determined using simulated-use tests. The requirements are further documented in Pesticide Assessment Guidelines Subdivision G: Product Performance 91-7(b).

PROPOSED EXPERIMENTAL STARTING DATE: Sept. 30, 1997
PROPOSED EXPERIMENTAL COMPLETION DATE: February 1, 1998
PROPOSED STUDY COMPLETION DATE: March, 1998

I. SUMMARY OF STUDY

The test materials will be tested for use-life using three product samples, each in a separate toilet, using the following conditions:

1. Toilet bowl cleaner placed in the toilet tank.
2. Toilets flushed 10 times/day/"x" weeks with non-chlorinated water supply.
3. Two water temperatures are used: 10-15°C (winter) and 25-30°C (summer.)
4. Toilet bowl water is sampled three times per week to determine pH and concentration of active.

Laboratory efficacy tests are conducted using samples of bowl water from the test toilets. Bacteriological assays will be conducted on treated and untreated samples that have been neutralized prior to standard plating procedures.

1. Samples of bowl water from three toilets with cleaner and corresponding untreated control samples from three toilets at 10-15°C (winter) are collected. Only this more stringent water temperature will be used to demonstrate efficacy.
2. The above samples are used to treat representative pathogenic gram-positive (*Streptococcus faecalis*) and gram-negative (*Salmonella choleraesuis*) with at least 10⁷ colony forming units per ml.
3. Microbial counts are conducted at a minimum of three exposure intervals, in addition to a "0-time" control.
4. Performance standard - the reduction of each test microorganism must be at least 99.9% over the "0-time" control and the parallel untreated inoculated control.

II. TEST SUBSTANCE

Name:	Clorox Automatic Toilet Bowl Cleaner (CATBC)
Formula No.:	F1997.0405
Batch/Lot No.:	6736-74-1
Sample Numbers:	6892-8-1 through 6892-8-9
Source:	CTC Pilot Plant
Product description:	White Tablet
Active:	Dantobrom
Active level:	81.1 %
Active CAS #:	89415-87-2

III. TEST SYSTEM IDENTIFICATION AND JUSTIFICATION**A. Test System**

1. *Streptococcus faecalis* ATCC 11700 represents a Gram-positive pathogenic bacteria.
2. *Salmonella chloraesus* ATCC 10708 represents a Gram-negative pathogenic bacteria.

B. Test System Justification - These organisms are specified in Pesticide Assessment Guidelines Subdivision G: Product Performance 91-7(b). The cultures will be purchased from American Type Culture Collection, rehydrated, and used in these studies. The new cultures will be assigned internal batch numbers by the microbiological contract laboratory. This batch number will be specified on the data sheets used to conduct these studies.

C. The organisms are transferred at least 3 times prior to testing. A 24-hour subculture from an agar surface is used to prepare the inoculum. The organisms are washed from the Tryptic Soy Agar surface with an appropriate amount of phosphate buffered water. The cultures are spectrophotometrically standardized to the desired bacterial density.

IV. EXPERIMENTAL**A TEST SUBSTANCE CHARACTERIZATION**

The test substance was characterized according to the analytical method SOP for active.

CHEMICAL ANALYSIS REQUIREMENTS

PROPERTY	LEVEL	SOP*
% Active Dantobrom	81.1 %	001-117-00

Statistical Analysis

The concentration of a component expressed as the mean of at least duplicate analyses.

$$\bar{x} = \frac{\sum x}{n}$$

The precision of a measurement is expressed as the concentration \pm one standard deviation (S).

$$S = \pm \sqrt{\frac{\sum (x - \bar{x})^2}{n-1}}$$

Where.

\bar{x} = mean concentration

x = concentration of individual observation

n = number of observations

S = standard deviation

B. SIMULATED-USE TESTS

1. The toilets used in this study are produced by American Standard and are the maximum 1.6 gallons per flush model. These will be standardized to 6 liters per flush by means of collecting and measuring the volume of discharge and adjusting the water level in the tank to yield 6 \pm 0.2 liters of drainage. This procedure is done prior to the start of testing. The water supply is filtered through packed charcoal to remove any halogen and then split into either the cold (10-15°C) system or the heated (25-30°C) system. A recirculating pump will create a continuous flow of water that will be supplied to each toilet at equal pressure and temperature. A calibrated thermocouple on the feed water line immediately following each water reservoir will be used to measure temperature. Each set of toilets will be set to flush automatically 10X/day by use of an air line that triggers an air cylinder to open the flushing mechanism in each toilet.
2. Halogen levels will be determined using a Hach DR/2000 Spectrophotometer. Halogen will be measured and calculated as total chlorine using Lonza SOP #10.7. This procedure is equivalent to USEPA method 330.5 for wastewater and Standard 4500-Cl G for drinking water. pH will be determined using Lonza SOP #10.8.

3. The simulated-use study begins when the cleaner is placed in the toilet tank and ends when the active level falls below the minimum use concentration that will be determined by the last laboratory efficacy test showing at least 99.9% reduction of each test organism.
4. Three tablets each will be tested at two water temperatures. Any remaining tablets to be returned to Study Director.

C. LABORATORY EFFICACY TESTING

Determine the efficacy of toilet bowl water containing residuals of the test substance against representative pathogenic Gram-positive and Gram-negative bacteria relative to the untreated control. Testing will start within four weeks after the introduction of the test substance into the toilet tank and continue at least weekly until failure.

1. A sufficiently large sample of toilet bowl water is aseptically removed from each of the treated 10-15°C toilets and from 3 untreated toilets after the toilets have been flushed and have stopped running (refilling the tank). The sample is removed from the center of the toilet bowl with a large pipet and placed into a sterile container, with minimum headspace. The container is immediately placed in a cooler that is chilled with ice or with synthetic ice packs. The cooler is transported immediately to the microbiological testing facility. Testing is conducted within 4 hours of sampling toilet water.

Aliquots are removed from this container for halogen level and pH determination concurrently with microbiological testing. Halogen levels will be determined using a Hach DR/2000 Spectrophotometer. Halogen will be measured and calculated as total chlorine using Benchmark SOP #I04002. This procedure is equivalent to USEPA method 330.5 for wastewater and Standard 4500-Cl G for drinking water. pH will be determined using Benchmark SOP #MB00401.

2. Two 99 ml aliquots will be removed from each treated and untreated toilet bowl water sample and placed into sterile flasks. The flasks and its contents are equilibrated in an incubator at 10-15°C for at least 20 minutes prior to testing.

3. Each sample is challenged at staggered intervals with 1 ± 0.1 ml of a standardized inoculum to achieve no less than 10^5 colony-forming units per ml (cfu/ml) as a final microbial level. The liquid in the flask is stirred and the inoculum is added to the swirling liquid. The flask is returned to the water bath until the next sampling time. The contents of the flask are stirred constantly through the exposure time.

4. Sampling

- a. **UNTREATED CONTROL**

At 0-time, 1, 5 and 10, 30 and 60 minutes ± 10 seconds, 1 ± 0.02 ml aliquots are removed from each untreated control flask and placed into 9 ± 0.1 mls of phosphate buffered water containing sufficient sodium thiosulfate to neutralize the halogen. The samples are serially diluted in Phosphate buffered water. Duplicate 1 ± 0.1 ml and 0.1 ± 0.02 ml aliquots of the appropriate dilutions are plated in Tryptic Soy Agar by the pour plate method.

- b. **TREATED SAMPLES**

After 1, 5 and 10, 30 and 60 minutes ± 10 seconds of exposure with the treated water, 1 ± 0.01 ml aliquots are removed from each flask and placed into 9 ± 0.1 mls of phosphate buffered water containing sufficient sodium thiosulfate to neutralize the halogen. Duplicate 1 ± 0.01 ml and 0.1 ± 0.02 ml aliquots are plated in Tryptic Soy Agar by the pour plate method.

- c. **NEUTRALIZER TOXICITY CONTROL**

At 0-time, 1 ± 0.02 ml of the standardized inoculum is mixed with 99 mls of Phosphate buffered water, serially diluted and plated in Tryptic Soy Agar by the pour plate method. This control is included as a neutralizer toxicity check.

5. All plates are incubated at $36 \pm 1^\circ\text{C}$ for 48 ± 6 hrs; *Streptococcus faecalis* may require an additional 24 hours for adequate colony growth.

The colonies on plates containing between 25 and 250 colonies are counted and averaged for each sample at each exposure time. If counts are more than 250, count CFU in one quarter of the plate that are representative of colony distribution and calculate the CFU/ml based on that dilution. If count on one quarter of plate is greater than 250, do not count the plate. If only one plate for a dilution has a count between 25 and 250, average the CFU from the two plates and calculate the CFU/ml based on that average. If plates from all dilutions have no colonies, report the CFU/ml <1 times the corresponding lowest dilution used. When colonies on duplicate plates and/or consecutive dilutions are counted, the results are averaged; however, if the average is below the lowest dilution used, the average is expressed as $<$ the lowest dilution.

6. The percent reduction over the control is calculated by the following equation:

$$\% \text{ Reduction} = \frac{[\text{average Control counts} - \text{average Treated counts}]}{\text{average Control counts}} \times 100$$

7. Performance Standard

The reduction of each test organism must be at least 99.9% over the "0-time" control and the parallel untreated inoculated control.

The minimum use concentration will be defined for each exposure period as the concentration of active at the last laboratory efficacy test showing at least 99.9% reduction of each test organism.

NEUTRALIZATION CHALLENGE

1. A 1 ± 0.01 ml aliquot of the treated water is mixed with 9 ± 0.01 mls of Phosphate buffered water containing sufficient sodium thiosulfate to neutralize the halogen. This is then challenged with approximately 10-100 cfu/ml for each microorganism. A 1 ± 0.1 ml aliquot is plated on TSA by pour plate method.
2. The same procedure is repeated with the untreated water.
3. Prior to inoculation, the concentration of each test organism is adjusted based on optical density and serially diluted in Phosphate buffered water. The neutralization challenge inoculum concentration is verified by plating on TSA to determine the original microbial level.

V. RECORDS

All reports and documentation pertaining to this study will be maintained as required by 40 CFR 160.195. Original raw data will be archived at the Clorox Services Company. Retain samples of the test substance will be maintained at the Clorox Services Company for, at a minimum, the period required by 40 CFR 160.195.

VI. NON-APPLICABLE GLP STANDARDS

GLP Standards §160.81(b)(7)&(9) do not apply to this study.

VII. PRINCIPAL INVESTIGATORS

<u>Name</u>	<u>Title</u>	<u>Responsibility</u>
Joseph Scheblein	Chemist	Toilet lab testing
Stephanie Olexa	President	Micro efficacy testing

All technical and scientific decisions regarding the conduct of this study should be confirmed with the Study Director.

VIII. APPROVAL

Approved By:



Date:

9-25-97

Study Director
Jim Rader, B.S. Chemistry
Home Cleaning Department
Clorox Services Company

Approved By:



Date:

9/25/97

Study Sponsor
Francie Mitchell, M.S. Chemistry
Manager, Home Cleaning Department
Clorox Services Company

AMENDMENT TO PROTOCOL

STUDY NUMBER: ~~6892-10~~ 5813-EX-093AMENDMENT # ~~5813-EX-093~~ 1

(Sanitizing Properties of Automatic Toilet Bowl Cleaner)

CHANGE:

Location and Description of the Change:

III A. Test System - Adding 4 test organisms (as requested by the Study Director).

C. Growth and harvesting of fungal test organisms added.

A. The schedule of organisms to be tested will be specified by Study Director on a weekly basis.

Protocol Change:

III A. Test System.

Add	#3	Staphylococcus aureus	ATCC 6538
	#4	Escherichia coli	ATCC 11229
	#5	Aspergillus mger	ATCC 16404
	#6	Candida albicans	ATCC 10231

C. Add Aspergillus niger and Candida albicans are grown on Sabouraud Dextrose Agar for 405 days at 20-25°C. Organisms are harvested by scraping surface growth and resuspending with phosphate buffer-H₂O. Concentration is verified by plate count method.

REASON FOR CHANGE.

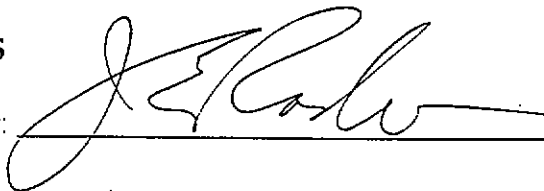
Sponsor requested additional organisms to be tested.

EFFECT OF CHANGE ON THE STUDY:

This amendment was developed on-site at Benchmark in order to address time constraints. A typed version of this amendment will be issued at a later date.

APPROVALS

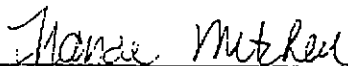
Study Director:



Date:

11/6/97

Study Sponsor:



Date:

12/2/97

AMENDMENT TO PROTOCOL**STUDY NUMBER:** 5813-EX-093**AMENDMENT #2****Title:** Sanitizing Properties of Automatic Toilet Bowl Cleaner**CHANGE:**

The purpose of this amendment is to provide for a provision in the frequency of toilet bowl water pulls for efficacy testing from weekly to an "as appropriate basis."

REASON FOR CHANGE:

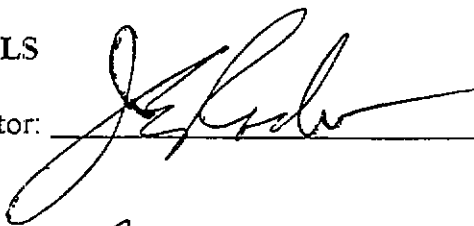
The weekly halogen levels are high enough that it is not necessary to conduct efficacy tests weekly. This amendment allows flexibility in reducing the number of pulls to help reduce costs and allows flexibility to work around Thanksgiving week or other holidays that come up during the duration of this study.

EFFECT OF CHANGE ON THE STUDY:

This change will not have an affect on the outcome of the study. The study will still identify the sanitizing properties of the automatic toilet bowl cleaner.

APPROVALS

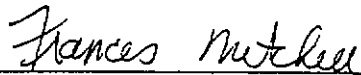
Study Director:



Date:

11/18/97

Study Sponsor:



Date:

11/19/97

AMENDMENT TO PROTOCOL

STUDY NUMBER: 5813-EX-093

AMENDMENT # 3

Sanitizing Properties of Automatic Toilet Bowl Cleaner

CHANGE:

(1) On page 6 of 10 of the protocol, under C. LABORATORY EFFICACY TESTING, #1 states that "Testing is conducted within 4 hours of sampling toilet water". This part is to be changed to "Testing is conducted within 8 hours of sampling toilet water."

(2) On page 7 of 10 of the protocol, under 4b. TREATED SAMPLES, it says "...aliquots are plated in Tryptic Soy Agar by the pour plate method." This part should be revised to say "The plates can be poured within 60 minutes of plate inoculation, provided the diluent is changed from Butterfield's phosphate buffer diluent to peptone-water (0.1% peptone, final pH 6.8)."

(3) On page 7 of 10, the protocol states that untreated control and treated samples will be tested at 1, 5, 10, 30, and 60 minutes exposure times. Certain exposure times may not be tested after awhile, depending on what results look like from a previous pull. If results fail at a particular exposure time, for any one particular organism or organisms, that exposure time will be eliminated from further testing for the specific organism or organisms.

REASON FOR CHANGE:

(1) This change was made in order to accommodate extended time for testing due to requesting the testing of additional of test organisms.

(2) This addition was made in order to be flexible about when agar medium is poured in order to accommodate the time needed to test the additional organisms.

(3) This change was made in order to streamline the work load.

EFFECT OF CHANGE ON THE STUDY:

The changes provided for by this amendment will not affect the outcome of this study. The study will still identify the sanitizing properties of the automatic toilet bowl cleaner.

APPROVALS

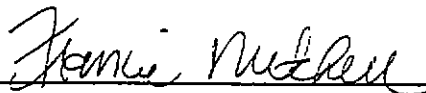
Study Director:



Date:

4/28/97

Study Sponsor:



Date:

4/28/97

AMENDMENT TO PROTOCOL

STUDY NUMBER: 5813-EX-093
AMENDMENT # 4
Sanitizing Properties of Automatic Toilet Bowl Cleaner

CHANGE:

Efficacy testing on toilet bowl water samples will be discontinued February 2.

REASON FOR CHANGE:

Although failure has not yet been demonstrated, the levels of total available chlorine in the test substance toilet bowl water samples have decreased to a level at which efficacy is no longer anticipated.

EFFECT OF CHANGE ON THE STUDY:

The experimental duration of the study could be slightly decreased. The overall results of the study will not be affected.

APPROVALS

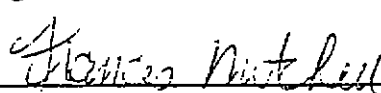
Study Director:



Date:

2/2/98

Study Sponsor:



Date:

2/2/98